

MPLA shows attenuated pro-inflammatory properties and diminished capacity to activate mast cells in comparison to LPS

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Abstract

Background: Monophosphoryl lipid A (MPLA), a non-toxic TLR4 ligand derived from lipopolysaccharide (LPS), is used clinically as an adjuvant in cancer, hepatitis, and malaria vaccines and in allergen-specific immunotherapy. Nevertheless, its cell activating effects have not been analyzed in a comprehensive direct comparison including a wide range of different immune cells. Therefore, the objective of this study was the side-by-side comparison of the immune modulating properties of MPLA and LPS on different immune cells.

Methods: Immune activating properties of MPLA and LPS were compared in human monocytes and mast cells (MCs), a mouse endotoxin shock model (ESM), and mouse bone marrow-(BM-) derived myeloid dendritic cells (mDCs), T cells (TCs), B cells, and MCs.

Results: In a mouse *in vivo* ESM and a human *ex vivo* monocyte activation test (MAT), MPLA induced the same cytokine secretion pattern as LPS (ESM: IL-6, IL-12, TNF- α ; MAT: IL-1 β , IL-6, TNF- α), albeit at lower levels. Mouse mDCs and *ex vivo* isolated B cells stimulated with MPLA required a higher threshold to induce Trif-dependent cytokine secretion (IL-1 β , IL-6, IL-10 and TNF- α) than did LPS-stimulated cells. In mDC:DO11.10 CD4 TC co-cultures, stimulation with MPLA, but not LPS resulted in enhanced OVA-specific IL-4 and IL-5 secretion from DO11.10 CD4 TCs. Unexpectedly, in both human and mouse MCs, MPLA, unlike LPS, did not elicit secretion of pro-inflammatory cytokines.

Conclusions: Compared to LPS, MPLA induced a qualitatively similar, but less potent pro-inflammatory immune response, but was unable to activate human or mouse MCs.

Key words: Adjuvant, Allergy, LPS, Mast Cells, MPLA;

Introduction

Allergen immunotherapy (AIT) with allergen extracts can lead to unwanted side effects and can be a multi-year ordeal for patients (1). Increasingly, however, AIT has been moving towards the use of optimized products to increase efficacy, while decreasing unwanted adverse-effects (2). Therefore, novel vaccine candidates and accompanying adjuvants have become an important area of investigation.

Ideal adjuvants should enhance long lasting antibody- and/or cell mediated-immunity to an antigen and reduce the quantity of antigen and overall number of vaccinations in a regimen, while being safe (3). Some currently licensed adjuvant preparations include oil emulsions, liposomes, aluminum salt derivatives (alum), innate immune agonists such as Monophosphoryl Lipid A (MPLA) or complex combinations of these (4,5). In order to design

improved therapeutics, it is important to deepen our knowledge of the mechanisms and cell types involved in the immune modulating capacities of such adjuvants.

MPLA is derived from the lipopolysaccharide (LPS) from *Salmonella minnesota* R595, by a series of organic extractions followed by mild acid and alkaline treatments (6). Although several lipid A species have been developed and tested as adjuvants, only MPLA has been evaluated in published human clinical trials where it has been administered to more than 300,000 human subjects (7). Several vaccines containing MPLA have been licensed or are in phase III trials including Fendrix[®] (hepatitis B), Cervarix[®] (human papillomavirus-16 and -18), and RTS,S[®] (malaria) (8-10). In the field of AIT, MPLA absorbed onto particulate aluminum salts (AS04) as part of the Pollinex[®] Quattro formulation is currently in clinical trials for the treatment of inhalant pollen allergies (11). Unlike the adjuvant aluminium hydroxide (alum), which promotes T-helper cell 2 (TH2) responses, MPLA has been shown to predominantly induce TH1-biased immune responses in mice (12). Furthermore, allergen therapeutics containing MPLA show a strong isotype switch from serum allergen-specific IgE antibodies towards IgG₁- and IgG₄-dominated responses in humans (13).

In vaccine formulations MPLA is usually applied as part of Adjuvant Systems (AS), which are complex mixtures consisting either of liposome-based formulations of antigen, MPLA and QS-21 (AS01), emulsion-based formulations of antigen, MPLA and QS-21 (AS02), or antigen and MPLA absorbed onto particulate aluminum salts (AS04) (11,14). Many studies have been performed using the different Adjuvant Systems (reviewed in (14)), but due to the complex nature of these mixtures the contribution of MPLA to the observed adjuvant effects is difficult to extrapolate.

A reduced secretion of pro-inflammatory cytokines by MPLA compared to LPS has been reported upon *in vivo* application (15), and *in vitro* using mouse peritoneal macrophages (16,17) and RAW 264.7 cells (18). Nevertheless, a comprehensive comparison of the

immune activating capacity of MPLA and LPS, on the cells which may contribute to MPLA's adjuvant activity, has not been published. Furthermore, at this point it is unclear whether MPLA's reduced toxicity is a result of the reduced secretion of pro-inflammatory cytokines (15,17,18) or, as we hypothesized, whether MPLA is unable to activate certain immune cell types that may contribute to adjuvant-induced toxicities.

Therefore, this study's objective was to compare comprehensively the effects of MPLA and its parent molecule LPS on a wide variety of immune cells in order to identify key differences in MPLA-induced immune activation. To this end, we determined the potential of MPLA and LPS to induce pro-inflammatory cytokine secretion from human monocytes and in a mouse endotoxin shock model. To further analyze the mechanisms and the cell types involved in MPLA-induced immune activation, we compared MPLA and LPS for their ability to activate mouse epithelial cells, BMDCs, and B cells, as well as mouse and human mast cells. Furthermore, we stimulated mDC:TC co-cultures with the mixture of both endotoxins with Ova, respectively, and analyzed the induction of allergen-specific TH1- and TH2-adaptive responses *in vitro*.

Methods

Antigens and Endotoxins

Endotoxin-free Ovalbumin, MPLA, and LPS were obtained from Hyglos (Bernried, Germany), Invivogen (Toulouse, France), or Sigma (Steinheim, Germany), respectively.

Mice and *in vivo* administration of LPS and MPLA

All animals were bred under specific pathogen-free conditions. I.p. administrations of 100 µg LPS or MPLA or PBS (used to dilute both endotoxins) as negative control were performed using C57BL/6 and TLR4^{-/-} mice (n=4) according to (19). All animal experiments were performed in compliance with approved protocols of the McGill University Animal Care Committee.

Immune cell generation and activation tests

Monocyte activation tests (MAT) were performed as previously described (20,21) in a total volume of 250 μ l. Written informed consent was obtained from all blood donors. Mouse myeloid dendritic cells (mDCs) were generated as described previously (22). B cells were isolated from spleens of naïve C57BL/6 mice using the Mouse B cell Isolation Kit (Miltenyi Biotech, Bergisch-Gladbach, Germany). Human mast cells (MCs) were differentiated for 10 weeks according to (23). C57BL/6 bone marrow cells were differentiated into bone marrow-derived cultured MCs (BMCMCs) by culture in 20% WEHI-3-conditioned CMLESS medium for 6 weeks. Mouse (LA-4) lung epithelial cells, BALB/c, C57BL/6, MyD88^{-/-}, Trif^{-/-}, and MyD88^{-/-}Trif^{-/-} mDCs, B cells, BMCMCs, or human MCs were stimulated with MPLA or LPS for 24 h. B cell viability was assessed using the Fixable Viability Dye eFlour[®] 450 (eBiosciences), human monocyte and MC viability by propidium iodide (Life Technologies, Carlsbad, CA USA) incorporation 24 h post-stimulation. Flow cytometric analysis and mDC:CD4 TC co-cultures were performed according to (24). Co-cultures were stimulated with MPLA or LPS in the presence or absence of 20 μ g/mL Ova for 72 h. The influence of LPS and MPLA on degranulation of anti-DNP IgE-sensitized (2 μ g/mL, (25)) BMCMCs was analyzed according to (26). Detection of phosphorylated Erk1/2 in BMCMCs (stimulated as indicated for 5 minutes at 37°C and 5% CO₂) was performed using an AF647-conjugated anti-pErk1/2 (Thr202/Tyr204) antibody (Cell Signalling Technologies, Danvers, MA) for 2 h. For pERK1/2 analysis, BMCMC preparations were gated on CD117-positive MCs.

ELISA

Cell supernatants were analyzed for the indicated cytokines or chemokines using the Ready-SET-Go! ELISA kits (eBiosciences, Frankfurt, Germany), DuoSet ELISA kits (R&D Systems), BD OptEIA ELISA sets (BD Biosciences), or the BD CBA human cytokine flex set (BD Biosciences) according to the manufacturers' recommendations.

Statistical analysis

Statistical analysis was performed with SAS[®]/STAT software, version 9.3, either using a paired t-test (Fig. 1A, Fig. 2A, Repository Fig. 1), a paired t-test with confidence intervals adjusted for multiple comparisons according to Bonferroni (Fig. 6A&B), or a Mann-Whitney test assuming an unpaired experimental design and a non-Gaussian distribution of values (Fig. 1B). Testing for a difference in linear dose-response relationship between LPS and MPLA in Figs. 3, 4, and 5 was performed assuming a linear relationship between the different doses, fitting a linear regression model to the data, and testing for differences in slopes based on the assumption of different intercepts of their respective curves.

Results

MPLA and LPS induce pro-inflammatory cytokines from human monocytes and in an *in vivo* mouse endotoxin shock model

To investigate the potency and safety of MPLA as an adjuvant, we determined the induction of pro-inflammatory cytokines from human blood monocytes (Fig. 1A). Whole blood cells from both non-allergic (NA) and birch pollen-allergic (A) donors were stimulated with LPS or MPLA. At high doses (≥ 2.5 $\mu\text{g/mL}$), MPLA induced a robust pro-inflammatory cytokine profile (IL-1 β , IL-6 and TNF- α) in all investigated donors, but at levels lower than that of LPS (Fig. 1A and data not shown). In the applied concentrations no cytotoxicity was observed for either LPS or MPLA (data not shown). Moreover, no differences in cytokine production were observed between non-allergic and birch pollen-allergic donors for either endotoxin (Fig. 1A).

To relate these results to a mouse system, we used a mouse septic shock model (19), in which C57BL/6 mice were injected i.p. with 100 μg of MPLA or LPS and serum was collected three hours post-injection. In accordance with the human monocyte activation test, MPLA induced the same set of cytokines (IL-12p40, TNF- α , and IL-6) as LPS, but again at significantly lower levels (Fig. 1B). Furthermore, MPLA-induced cytokine production was abrogated in TLR4-deficient mice (Fig. 1B).

High dose MPLA or LPS induce comparable epithelial and dendritic cell activation

To investigate the cell types responsible for pro-inflammatory cytokine production and the underlying mechanisms we stimulated the mouse lung epithelial cell line LA-4 with MPLA and LPS (Fig. 2A). Epithelial cells form barriers, are usually the first cells that come into contact with invading pathogens, and orchestrate downstream immune responses via the production of chemokines or damage associated molecular patterns (DAMPs). Initial secretion of the myeloid chemoattractant CCL2 was observed at concentrations of >10 ng/ml MPLA and >1 ng/ml LPS with LPS showing a 2 to 8-fold higher potency for CCL2-induction (Fig. 2A).

Next we investigated the effects of MPLA and LPS on C57BL/6-derived bone marrow derived myeloid dendritic cells (mDCs), which are attracted by CCL2. In high concentrations (>1 µg/mL) MPLA induced identical levels of CD40 (Fig. 2B) and CD69 (Repository Fig. 2) expression compared to LPS; however, initial LPS-induced activation required only 1 ng/mL of endotoxin compared to 100 ng/mL for MPLA (Fig. 2B). These results were paralleled by the cytokine secretion profile of stimulated mDCs where MPLA and LPS induced the same set of cytokines (IL-1 β , IL-10 and TNF- α) but approx. 100- to 1000-fold higher amounts of MPLA were required than for LPS (Fig. 2C). Of note, IL-1 β secretion induced by 10 µg/mL of MPLA was about 4.6-fold higher than observed for the respective dose of LPS (Fig. 2C). These results were confirmed using BABL/c-derived mDCs (Repository Fig. 1).

When investigating the expression of co-inhibitory molecules, which provide tolerogenic signals to T cells, we could not detect B7-DC expression upon stimulation with either LPS or MPLA (Repository Fig. 2). In contrast to this, each of the endotoxins increased surface expression levels of B7-H1 to the same degree (Repository Fig. 2). Finally, when mDC preparations were stimulated with higher endotoxin concentrations (10 µg/ml and 1 µg/ml) LPS induced a stronger upregulation of B7-H4 expression, whereas in concentrations lower than 1 µg/ml no difference between LPS- and MPLA-induced B7-H4 expression could be

detected (Repository Fig. 2). Here, the threshold for mDC activation was shown to be 10 ng/ml of either endotoxin (Repository Fig. 2).

MPLA-induced cytokine production is partially Trif-dependent in mDCs

To investigate the mechanism of MPLA-induced cytokine secretion, mDCs differentiated from C57BL/6, MyD88^{-/-}, Trif^{-/-}, and MyD88^{-/-}Trif^{-/-} mice were used. Endotoxin-induced anti-inflammatory IL-10 production was completely abrogated in MyD88^{-/-}, Trif^{-/-}, or MyD88^{-/-}Trif^{-/-} cells (Fig. 3). However, pro-inflammatory IL-1 β , IL-6 and TNF- α secretion were abrogated in either MyD88^{-/-} or MyD88^{-/-}Trif^{-/-} cells, but not in Trif-deficient cells (Fig. 3). Remarkably, MPLA-induced IL-1 β , IL-6 and TNF- α secretion was significantly reduced but not abolished in Trif^{-/-} mDCs (Fig. 3, p-values for differences in slope intercepts between LPS and MPLA curves in Trif^{-/-} mDC: TNF- α : p<0.0001, IL-6: p<0.0001, IL-1 β : p=0.0001). In contrast, LPS-induced IL-6 and TNF- α secretion showed slight reductions compared to wild-type cells at lower concentrations ($\leq$ 100 ng/mL, Fig. 3).

MPLA and LPS show a similar activation capacity and cytotoxicity on ex vivo isolated B cells

After characterizing the capacity of MPLA to initiate innate immune responses, we next investigated the effects of MPLA-stimulation on the major effector cells of the adaptive immune system (Fig. 4 & Fig. 5). Interestingly, using splenic B cells from naïve C57BL/6 mice (purity $\geq$ 95% Fig. 4), each endotoxin readily induced IL-10 secretion with high dose LPS (>100 ng/ml) being significantly more potent in doing so (Fig. 4B) whereas no TNF- α secretion could be detected (data not shown). Moreover, MPLA readily induced CD69 expression on B cells, but at lower levels than LPS (Fig. 4C). Since we observed IL-10 secretion from endotoxin-stimulated naïve B cells we also checked for the expression of co-inhibitory molecules upon endotoxin treatment. Here, LPS induced a strong upregulation of B7-H1 while MPLA-induced B7-H1 expression was much lower (Fig. 4C). Moreover, we were able to detect an increase in PD-1 expression on stimulated B cells with again LPS being

more potent than MPLA (Fig. 4C). However, MPLA-induced cytotoxicity was comparable to that induced by LPS, showing cell death at high doses of MPLA or LPS (≥ 100 ng/mL, Fig. 4D).

MPLA but not LPS boosts Ova-specific TH2-cytokine production

The impact of MPLA- or LPS-induced APC activation on the differentiation of antigen-specific T cells was investigated using BALB/c mDCs:DO11.10 CD4⁺ T cell co-cultures stimulated with MPLA or LPS, in the presence or absence of Ova. Each endotoxin boosted dose-dependent production of mDC-derived IL-10, correlating with a suppression of Ova-specific IFN- γ and IL-2 secretion (Fig. 5). Interestingly, co-stimulation with MPLA, but not with LPS, enhanced Ova-induced IL-4 and IL-5 secretion (Fig. 5, $p = 0.0111$ of slope intercepts at 20 μ g/mL Ova). However, low-dose MPLA co-stimulation had no effect on Ova-induced IL-13 secretion, which was readily suppressed upon LPS co-administration (Fig. 5).

In contrast to LPS, MPLA is unable to activate mast cells

To complete our analysis, we compared MPLA and LPS for their ability to activate MCs, which can function both as important sentinels of the immune system and as harmful effector cells of allergic responses. For this we used both *in vitro* differentiated human MCs (Fig. 6A) and mouse BMCMCs (Fig. 6B). Interestingly, LPS, but not MPLA, stimulation resulted in a considerable IL-8 release from human MCs (Fig. 6A) and dose-dependent IL-6 release from mouse BMCMCs (Fig. 6B). Neither LPS nor MPLA induced IL-13, GM-CSF or MIP-1 β secretion from human MCs (Repository Fig. 3). Even when applied in high concentrations, no degranulation of anti-DNP-IgE-sensitized MCs was detected upon stimulation with either endotoxin (Fig. 6C) and neither MPLA nor LPS had any effect on IgE- and DNP-induced β -hexosaminidase release (Fig. 6C). Analysis of BMCMC viability excluded the possibility that the lack of IL-6 secretion upon stimulation with MPLA was due to cytotoxicity of MPLA (Fig. 6D). Moreover, no upregulation of TLR4 was detected on LPS- or MPLA-stimulated BMCMCs (data not shown). Mechanistically, compared to unstimulated controls, stimulation

of BMCMCs with LPS, but not with MPLA, resulted in increased levels of ERK1/2 phosphorylation (Fig. 6E), consistent with the induction of IL-6 secretion.

Discussion

The recent approval of several vaccine formulations adjuvanted with MPLA underlines the importance of conducting further investigations into the immune mechanisms and the target cells contributing to MPLA's adjuvant effects. Direct comparison of the immune-modulating properties of MPLA to its parent molecule LPS can be helpful in elucidating these mechanisms; however, most such studies have been limited in scope. These studies focused either on the use of MPLA in complex Adjuvant Systems, performed general *in vivo* analyses, or studied single cell types *in vitro*. Therefore, we used a wide variety of mouse and human model systems to perform an extensive comparison of the innate and adaptive immune cell-activating potential of these endotoxins.

In our endotoxin shock model, MPLA administered i.p. at a high dose (100 µg) induced lower levels of IL-6, IL-12p40 and TNF-α than did LPS; however, MPLA still caused a significant, TLR4-dependent, production of these pro-inflammatory cytokines. Moreover, using a validated human blood monocyte pyrogenicity test (20,21), we found that monocytes from both allergic or non-allergic donors challenged with 25 µg of MPLA or LPS produced similar levels of IL-1β, IL-6 and TNF-α. These results provide evidence that, although MPLA is considered safe and non-toxic, in high doses it can still invoke high levels of strongly pyrogenic, pro-inflammatory cytokines such as IL-1β and IL-6.

Using well-established mouse cell culture models, we examined the modulation of specific innate and adaptive immune cell types by MPLA treatment. Previous work has investigated the effect of MPLA on a variety of immune cells of myeloid and lymphoid origin (16,27,28). We have taken these studies further and demonstrated that MPLA activates *in vitro* and *ex vivo* mouse cell cultures of epithelial cells, dendritic cells, TCs, and BCs, when applied in

high concentrations ($>1 \mu\text{g/mL}$), much in the same manner as LPS. To our knowledge, this is the first demonstration of MPLA-activated epithelial cells to produce CCL2.

Furthermore, we found, that MPLA-induced secretion of the anti-inflammatory cytokine IL-10 from splenic BCs, which if it occurs *in vivo*, might have beneficial effects when MPLA-containing vaccines are used for the treatment of allergic diseases. Additionally, both endotoxins influenced expression levels of co-inhibitory molecules on naïve B cells. For both B7-H1 and PD-1, LPS-induced upregulation was more pronounced whereas MPLA induced only minor increases. In this context, PD-1 was shown to be expressed on major human B-cell subsets isolated from peripheral blood and lymph nodes and to be recruited to the B cell receptor upon TLR9 triggering (29). Furthermore, expression of PD-1 ligands was also shown to be induced by TLR9 activation, suggesting that PD-1/PD-L complexes can act as inhibitors of the B cell activation cascade (29).

In line with this, the increased expression of co-inhibitory molecules on LPS or MPLA stimulated mDCs is especially interesting in the context of the observed T cell derived cytokine secretion in LPS/MPLA plus Ova co-stimulated co-cultures (Fig. 5). Here, the observed increase in surface expression of the co-inhibitory molecules B7-H1 and B7-H4 did correspond to the partial, endotoxin-induced, suppression of T cell-derived cytokine secretion (IFN- γ for both endotoxins, IL-4 and IL-13 only for LPS) when high amounts of endotoxins were co-administered (Fig. 5).

By contrast, our studies of MPLA+Ova or LPS+Ova stimulated co-cultures of Ova-specific CD4⁺ T cells showed a potentially worrisome finding in the context of allergic disorders, in that low doses of MPLA+Ova caused induction of the TH2 cytokines IL-4 and IL-5 (but not IL-13) from Ova-specific CD4⁺ T cells, whereas LPS+Ova stimulation did not. This finding is likely explained by the reduced induction of immune-suppressive IL-10 by low dose MPLA-stimulation and points to the possibility that when co-applied with antigen, MPLA may have

the capacity to cause or enhance TH2-biased immune responses via effects on IL-4 and IL-5 production. However, the net effect of MPLA used in the setting of allergic disorders may depend on the final balance between the enhanced secretion of anti-inflammatory IL-10 and the possible enhancement of TH2 immune responses due to increased production of IL-4 and IL-5.

LPS-induced, innate TLR4-dependent signaling proceeds via the adaptor molecule MyD88, and has a weaker dependence on the adaptor Trif (30), whereas MPLA was proposed to have an inherent bias towards signaling via Trif (16). We confirmed MPLA's Trif-dependency in mDCs and added new knowledge by showing that the production of IL-10 by mDCs was almost completely abrogated in Trif^{-/-} mDCs.

Finally, the analysis of MC activation confirmed our initial hypothesis that MPLAs reduced toxicity may be in part caused by its inability to activate certain types of immune cells. Here, in contrast to LPS, MPLA did not induce activation of either human MCs or mouse BMCMCs. Concordantly, stimulation of BMCMCs with LPS, but not with MPLA, increased ERK1/2 phosphorylation, suggesting a lack of MC activation upon MPLA stimulation under the conditions tested. Whereas the potential of LPS to activate BMCMCs is well described in the literature, the effects of MPLA on MCs have so far not been investigated. Reports suggest that the limited response of MCs, in particular towards LPS is one of the mechanisms restraining the ability of LPS to elicit excessive host responses at host-environment interfaces (31). The reduced reactivity of MCs towards LPS was explained by the absence of the adaptor protein TRAM, which initiates Trif-mediated TLR4 signaling (31). Therefore, this lack of TRAM expression is likely to explain, at least in part, the absence of MC responses upon stimulation with the Trif-biased TLR4-ligand MPLA (Fig. 3 and (16)). These results are especially interesting since an inability of MPLA to activate MCs may be an important safety feature and is the first qualitative difference we observed when comparing it to its parent

molecule LPS. However, we can't rule out the possibility that MPLA might be able to directly or indirectly activate populations of MCs other than those studied in this report.

In summary, using *in vitro*, *ex vivo*, *in vivo* human and mouse test systems we added important knowledge regarding MPLA's adjuvant capacity, showing that MPLA-stimulation induced similar but attenuated immune activation in most immune cells (with MPLA's immune activation profile being similar to a strongly diluted LPS preparation), but failed to activate human or mouse MCs. Future studies should investigate whether the MPLA-induced pro-inflammatory cytokine secretion has detrimental effects and/or is critically necessary for the efficacy of MPLA-adjuvanted vaccines.

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Figure legends

Figure 1: Exposure to MPLA or LPS results in similar pro-inflammatory cytokine patterns from human monocytes and in a mouse *in vivo* endotoxin shock model.

Human monocyte activation test using blood from non-allergic (NA) and birch pollen-allergic (A) donors stimulated with 25 µg/well LPS (grey) or MPLA (black, **A**). Endotoxin-induced cytokine secretion in sera of C57BL/6 or TLR4^{-/-}C57BL/6 mice injected i.p. with 100 µg LPS or MPLA (**B**). Data are mean results of four blood donors or mice per group ± SD.

Figure 2: High-doses of MPLA or LPS induce similar levels of epithelial cell and DC activation and cytokine secretion. CCL2 secretion from LA-4 cells stimulated with LPS (grey) or MPLA (black, **A**). Activation (**B**) and cytokine secretion (**C**) of C57BL/6 mDCs stimulated for 24 h with LPS (**B**: black, **C**: grey) or MPLA (**B**: orange, **C**: black). Data are

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mean results of four (**A**), representative from one out of three (**B**) and mean of three independent experiments $\pm$ SD (**C**).

Figure 3: MPLA-induced pro-inflammatory cytokine secretion from mDCs is MyD88 and, in part, Trif dependent. Cytokine secretion of C57BL/6, MyD88^{-/-}, Trif^{-/-} and MyD88^{-/-} Trif^{-/-} mDCs stimulated with LPS or MPLA for 24 h. Results are the mean $\pm$ SD of two independent experiments.

Figure 4: MPLA stimulation of naïve B cells results in activation, cytokine secretion and cytotoxicity comparable to LPS. Purity of B cells isolated from spleens of naïve C57BL/6 mice (**A**). IL-10 secretion (**B**), cell activation status (**C**) and viability (**D**, percentage of Fixable Viability Dye eFlour® 450-positive dead cells indicated in the respective overlays) of purified B cells stimulated with LPS or MPLA for 24 h. Data are representative results from one out of three (**C**) or two (**D**) independent experiments for (**A** + **C**) and mean of five independent experiments $\pm$ SD for (**B**).

Figure 5: MPLA, but not LPS, boosts Ova-specific IL-4 and IL-5 secretion with a concurrent repression of TH1 cytokine secretion by either endotoxin. Cytokine secretion of BALB/c mDCs co-cultured with DO11.10 CD4⁺ T cells and stimulated with MPLA (black) or LPS (grey) in the presence or absence of Ova (white) for 72 h. Data are mean results of two independent experiments $\pm$ SD.

Fig. 6: LPS, but not MPLA, stimulation results in mast cell activation, while neither endotoxin influences IgE- and DNP-induced degranulation. Pro-inflammatory cytokine secretion from LPS or MPLA stimulated human MCs (**A**) and C57BL/6 mouse BMCMCs (**B**). Degranulation of endotoxin-stimulated, anti-DNP IgE-sensitized C57BL/6 BMCMCs with or without DNP-HSA (**C**). Viability of stimulated BMCMCs (**D**). Phosphorylation of Erk1/2 in LPS or MPLA stimulated CD117⁺ BMCMCs (**E**). Data are mean results of two (**A**), three (**B** and **C**, **D**) or representative results (**E**) from three independent experiments $\pm$ SD. All endotoxin concentrations are given in μ g/mL.

References

1. Justicia JL, Cardona V, Guardia P, Ojeda P, Olaguíbel JM, Vega JM et al. Validation of the first treatment-specific questionnaire for the assessment of patient satisfaction with allergen-specific immunotherapy in allergic patients: the ESPIA questionnaire. *J Allergy Clin Immunol* 2013;**131**:1539–1546.
2. Valenta R. The future of antigen-specific immunotherapy of allergy. *Nat Rev Immunol* 2002;**2**:446–453.
3. Reed SG, Orr MT, Fox CB. Key roles of adjuvants in modern vaccines. *Nat Med* 2013;**19**:1597–1608.
4. Alving CR, Peachman KK, Rao M, Reed SG. Adjuvants for human vaccines. *Current Opinion in Immunology* 2012;**24**:310–315.
5. Fox CB, Baldwin SL, Duthie MS, Reed SG, Vedvick TS. Immunomodulatory and physical effects of oil composition in vaccine adjuvant emulsions. *Vaccine* 2011;**29**:9563–9572.
6. Ulrich JT, Myers KR. Monophosphoryl lipid A as an adjuvant. Past experiences and new directions. *Pharm Biotechnol* 1995;**6**:495–524.
7. Cluff CW. Monophosphoryl lipid A (MPL) as an adjuvant for anti-cancer vaccines: clinical results. *Adv Exp Med Biol* 2009;**667**:111–123.
8. Kundi M. New hepatitis B vaccine formulated with an improved adjuvant system. *Expert Rev Vaccines* 2007;**6**:133–140.
9. Agnandji ST, Lell B, Soulanoudjingar SS, Fernandes JF, Abossolo BP, Conzelmann C et al. First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. *N Engl J Med* 2011;**365**:1863–1875.
10. GlaxoSmithKline Vaccine HPV-007 Study Group, Romanowski B, de Borja PC, Naud PS, Roteli-Martins CM, De Carvalho NS et al. Sustained efficacy and immunogenicity of the human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine: analysis of a randomised placebo-controlled trial up to 6.4 years. *Lancet* 2009;**374**:1975–1985.
11. Patel P, Salapatek AMF. Pollinex Quattro: a novel and well-tolerated, ultra short-course allergy vaccine. *Expert Rev Vaccines* 2006;**5**:617–629.
12. Casella CR, Mitchell TC. Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. *Cell Mol Life Sci* 2008;**65**:3231–3240.
13. Mothes N, Heinzkill M, Drachenberg KJ, Sperr WR, Krauth MT, Majlesi Y et al. Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. *Clin Exp Allergy* 2003;**33**:1198–1208.
14. Garçon N, Van Mechelen M. Recent clinical experience with vaccines using MPL- and QS-21-containing adjuvant systems. *Expert Rev Vaccines* 2011;**10**:471–486.

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15. Henricson BE, Benjamin WR, Vogel SN. Differential cytokine induction by doses of lipopolysaccharide and monophosphoryl lipid A that result in equivalent early endotoxin tolerance. *Infection and Immunity* 1990;**58**:2429–2437.
 16. Casella CR, Mitchell TC, Mata-Haro V, Martin M, Chilton PM, Cekic C et al. The Vaccine Adjuvant Monophosphoryl Lipid A as a TRIF-Biased Agonist of TLR4. *Science* 2007;**316**:1628–1632.
 17. Okemoto K, Kawasaki K, Hanada K, Miura M, Nishijima M. A potent adjuvant monophosphoryl lipid A triggers various immune responses, but not secretion of IL-1beta or activation of caspase-1. *The Journal of Immunology* 2006;**176**:1203–1208.
 18. Aybay C, Imir T. Comparison of the effects of Salmonella minnesota Re595 lipopolysaccharide, lipid A and monophosphoryl lipid A on nitric oxide, TNF-alpha, and IL-6 induction from RAW 264.7 macrophages. *FEMS Immunol Med Microbiol* 1998;**22**:263–273.
 19. Vogel SN, Johnson D, Perera PY, Medvedev A, Larivière L, Qureshi ST et al. Cutting edge: functional characterization of the effect of the C3H/HeJ defect in mice that lack an Lpsn gene: in vivo evidence for a dominant negative mutation. *The Journal of Immunology* 1999;**162**:5666–5670.
 20. Hoffmann S, Peterbauer A, Schindler S, Fennrich S, Poole S, Mistry Y et al. International validation of novel pyrogen tests based on human monocytoic cells. *J Immunol Methods* 2005;**298**:161–173.
 21. Montag T, Spreitzer I, Löschner B, Unkelbach U, Flory E, Sanzenbacher R et al. Safety testing of cell-based medicinal products: opportunities for the monocyte activation test for pyrogens. *ALTEX* 2007;**24**:81–89.
 22. Schülke S, Waibler Z, Mende M-S, Zoccatelli G, Vieths S, Toda M et al. Fusion protein of TLR5-ligand and allergen potentiates activation and IL-10 secretion in murine myeloid DC. *Mol Immunol* 2010;**48**:341–350.
 23. Gaudenzio N, Laurent C, Valitutti S, Espinosa E. Human mast cells drive memory CD4+ T cells toward an inflammatory IL-22+ phenotype. *J Allergy Clin Immunol* 2013;**131**:1400–7.e1411.
 24. Schülke S, Wolfheimer S, Gadermaier G, Wangorsch A, Siebeneicher S, Briza P et al. Prevention of intestinal allergy in mice by rflaA:Ova is associated with enforced antigen processing and TLR5-dependent IL-10 secretion by mDC. *PLoS ONE* 2014;**9**:e87822.
 25. Hill PN, Liu FT. A sensitive enzyme-linked immunosorbent assay for the quantitation of antigen-specific murine immunoglobulin E. *J Immunol Methods* 1981;**45**:51–63.
 26. Tam S-Y, Tsai M, Snouwaert JN, Kalesnikoff J, Scherrer D, Nakae S et al. RabGEF1 is a negative regulator of mast cell activation and skin inflammation. *Nat Immunol* 2004;**5**:844–852.
 27. Didierlaurent AM, Morel S, Lockman L, Giannini SL, Bisteau M, Carlsen H et al. AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. *J Immunol*

2009;**183**:6186–6197.

28. Röver AC, Reimann S, Zuberbier T, Worm M. Immunophenotypic characterization of peripheral B cells. During short-term immunotherapy with tree pollen allergoid and the immunoadjuvant monophosphoryl lipid A. *J Invest Allergol Clin Immunol* 2002;**12**:227–234.
29. Thibult M-L, Mamessier E, Gertner-Dardenne J, Pastor S, Just-Landi S, Xerri L et al. PD-1 is a novel regulator of human B-cell activation. *Int Immunol* 2013;**25**:129–137.
30. Ahrens B, Freund T, Rha R-D, Dittrich A-M, Quarcoo D, Hutloff A et al. Lipopolysaccharide stimulation of dendritic cells induces interleukin-10 producing allergen-specific T cells in vitro but fails to prevent allergic airway disease. *Exp Lung Res* 2009;**35**:307–323.
31. Keck S, Müller I, Fejer G, Savic I, Tchaptchet S, Nielsen PJ et al. Absence of TRIF signaling in lipopolysaccharide-stimulated murine mast cells. *J Immunol* 2011;**186**:5478–5488.

Repository figure legends

Repository figure 1: BALB/c mDCs show substantial cytokine secretion when stimulated with MPLA. Cytokine secretion of BALB/c mDCs stimulated with LPS (grey) and MPLA (black) for 24 h. Data are mean results of three independent experiments $\pm$ SD.

Repository figure 2: High-dose MPLA or LPS induce similar levels of co-stimulatory and co-inhibitory molecules on stimulated mDCs. Expression levels of CD69, B7-DC, B7-H1 and B7-H4 were determined on C57BL/6 mDCs stimulated for 24 h with LPS (black) or MPLA (orange). Unstimulated: grey tinted, unstained cells: grey dotted. Data are representative results from one out of three independent experiments.

Repository figure 3: Neither LPS nor MPLA induces IL-13, GM-CSF or MIP-1 β secretion from human mast cells. Cytokine secretion from human mast cells stimulated with LPS (grey) and MPLA (black) over night. Data are mean results from 2 independent experiments $\pm$ SD.

















